EXHIBIT C

Experimental Chemotherapy

Chemotherapy

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Establishment and Characterization of Cisplatin-Resistant Human Epidermoid Carcinoma Cell Line, A431 Cell

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Key Words
A431
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Oral cancer

Abstract
Cisplatin, cis-diamminedichloroplatinum(II) (CDDP) is one of the most important anticancer agents, initially producing good responses in various tumors. However, resistance to this drug often develops in various tumors, and additional administration decreases its chemotherapeutic efficacy. The precise mechanism of acquisition of resistance to this drug is still uncertain. However in the present study, we established two CDDP-resistant sublines A431/CDDP1 and A431/CDDP2 from human epidermoid carcinoma cell line A431. These resistant sublines were constituted by exposing A431 cells to a gradually increasing dose of CDDP (A431/CDDP1), and by mutagenic induction with mutagen (A431/CDDP2). A431/CDDP1 and A431/CDDP2 have developed 3.1 and 2.7 times more resistance to CDDP than the original A431 cell in terms of IC_{50}. The two CDDP-resistant sublines showed cross-resistance to the CDDP analogue, carboplatin (CBDCA), but not to other chemotherapeutic drugs such as Adriamycin (ADR) and 5-fluorouracil (5-FU). These CDDP-resistant sublines were transplanted into nude mice to demonstrate the resistance to CDDP treatment in vivo. According to the in vitro assay, the mechanism of resistance in A431/CDDP1 and A431/CDDP2 seems to be based on a reduction of intracellular accumulation of CDDP, because their platinum concentration, which is the major component of CDDP, significantly declined. The established CDDP-resistant sublines may be used in further trials to improve the understanding of the mechanisms of resistance to CDDP.

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Introduction

Cisplatin, cis-diamminedichloroplatinum (II) (CDDP) possesses a potent anticancer activity [1]. In the treatment of head and neck cancer, CDDP has been most widely used as base of single and combination chemotherapy as well as radiosensitizer [2–4]. However, development of drug resistance is a major obstacle for the success of these cancer therapies. Although, it is of great importance to elucidate the resistance mechanism of CDDP to improve this clinical problem, the molecular basis of CDDP resistance has not been conclusively defined [5–7].

Regarding the mechanism of resistance to CDDP, several hypotheses have been advanced: protective and detoxification mechanisms, including decreased drug accumulation [8], increased content of intracellular thiols such as glutathione [9–11] and metallothioneins [12, 13], reduction of DNA cross-linking as a consequence of decreased drug accessibility to DNA, and/or increased repair [14, 15]. In most of the patients, the resistance to the anticancer agent has been reported to be only 3- to 6-fold [16, 17].

For determination of the mechanisms of resistance to CDDP, cell lines of leukemia [18] and ovarian carcinoma [19] have been exclusively used. However, there is little information about the resistance to CDDP in oral cancer due to the few established CDDP-resistant cell lines derived from head and neck epithelial carcinoma [20].

In the present study, we established two CDDP-resistant cell lines derived from human epidermoid carcinoma cell line A431, and examined their characteristics. Both cell lines showed low resistance to CDDP. In addition, they were transplanted into nude mice to develop an in vivo CDDP resistance model.

Materials and Methods

Anti-Cancer Drugs

The anti-cancer drugs used were: cisplatin (CDDP) from Nippon Kayaku, Tokyo, Japan; carboplatin (CBDCA) from Bristol Myers, New York, N.Y., USA; Adriamycin (ADM) and 5-fluorouracil (5-FU) from Kyowa Hakko, Tokyo, Japan.

Establishment of CDDP-Resistant A431 Cells

Human epidermoid carcinoma cell line A431 cells were cultured in DMEM/F-12 (Life Technologies, Inc., Rockville, Md., USA) containing 5% FBS (Life Technologies) and 1% penicillin-streptomycin at 37°C, 5% CO_2. A431 cells (2×10^5/60 mm dish) were grown and CDDP was then added to the medium at increasing concentrations of 0.2, 0.4, 0.5, 0.7, and 1.0 μg/ml of CDDP. During continuous exposure to CDDP, culture medium was replaced with freshly prepared medium containing CDDP at indicated concentrations every 3 days. Colonies survived when exposure to 1.0 μg/ml CDDP was selected, and the selected clone was named A431/CDDP1. A431 cells (1×10^6/90-mm dish) in 10 ml of medium were treated with 1 μ*M* of 1-methyl-3-nitro-1-nitrosoguanidine (MNNG; Aldrich Chemical Co., Milwaukeee, Wisc., USA) for 24 h, and then the cells were incubated in MNNG-free medium for 6 days. CDDP was then added to the medium at a concentration of 1.0 μg/ml, and incubated for 7 days. Colonies were isolated and the purified clone was named A431/CDDP2.

In vitro Drug Sensitivity Assay

The parent cell line A431 (A431/P) and CDDP-resistant cell line, A431/CDDP1 and A431/CDDP2 (1×10^4/well) were seeded in a 24-multiwell dish. After 24 h, various concentrations of CDDP and other chemotherapeutic agents (CBDCA, ADM and 5-fluorouracil; 5-FU) were added to the medium at the following dosages: CBDCA: 1×10^{-4}, 3×10^{-4}, 1×10^{-3}, 3×10^{-3}, 1×10^{-2}, 3×10^{-2}, 1×10^{-1} and 3×10^{-1} μg/ml; ADM: 1×10^{-6}, 3×10^{-6}, 1×10^{-5}, 3×10^{-5}, 1×10^{-4}, 3×10^{-4} and 1×10^{-3} μg/ml, 5-FU: 3×10^{-5}, 1×10^{-4}, 3×10^{-4}, 1×10^{-3}, 3×10^{-3}, 1×10^{-2}, 3×10^{-2} and 1×10^{-1} μg/ml). After an additional 3 days of culture, the viable cells were counted. All counts were done in triplicate, and the viability was assessed by the trypan blue dye exclusion assay.

In vivo Drug Sensitivity Assay

A431/P, A431/CDDP1 and A431/CDDP2 cells (1×10^7) were injected subcutaneously into 4- to 5-week old female BALB/c-*nu/nu* mice (CLEA Japan,

Tokyo, Japan) with a 27-gauge needle. The animals were housed in a pathogen-free environment under controlled conditions of light and humidity in our animal facility. CDDP (5 mg/kg body weight) was administered intraperitoneally once a week from 2 weeks after tumor inoculation. Tumor size was determined by recording maximum and minimum diameters with a caliper once a week. Tumor volume (V) was calculated by the formula $V = LW^2/2$, where L is the length and W is the width in millimeters of the tumor. Antitumor activity was evaluated by the mean tumor volume of a group of mice.

Measurement of Intracellular Platinum Concentration

We measured the platinum concentration, which is the major component of CDDP, to evaluate the accumulation of intracellular CDDP. In brief, the cells were adjusted to 2×10^6 cells/90-mm dish and cultured for 2 days. The cells were washed twice with EMEM (Kyokuto Seiyaku K.K., Tokyo, Japan) and incubated with CDDP (20 μg/ml) in EMEM for 1–7 h. After lysing these samples in 4 *N* HNO_3, platinum concentration was measured at 265.9 nm using the Zeeman Atomic Absorption Spectrometer (Z-800, Hitachi K.K., Tokyo, Japan) [21].

Statistical Analysis

The Mann-Whitney test was used to analyze the statistical differences between the parent A431 (A431/P) and the CDDP-resistant cell lines (A431/CDDP1 and A431/CDDP2).

Results

Characteristics of CDDP-Resistant A431 Cells

We established two CDDP-resistant A431 sublines, A431/CDDP1 and A431/CDDP2, by two methods, gradually increasing the doses of CDDP and the mutagenic induction with MNNG. Although CDDP inhibited the growth of A431/P, A431/CDDP1 and A431/CDDP2 cells in a dose-dependent manner, its inhibitory activity on A431/CDDP1 and A431/CDDP2 cell growth was less than that on A431/P cells (fig. 1). A431/CDDP1 and A431/CDDP2 have become 3.1 and 2.7 times more resistant to CDDP than the respective A431/P in terms of IC_{50} (table 1). The CDDP resistance in the sublines was stable over 8 months even in CDDP-free medium. The doubling times of A431/P, A431/CDDP1 and A431/CDDP2 were about 19 h; the differences among these cell lines were not statistically significant (data not shown).

Table 1. Characteristics of CDDP-resistant sublines

	IC_{50}	Relative resistance
A431/P	0.096	1.0
A431/CDDP1	0.30	3.1
A431/CDDP2	0.26	2.7

Relative resistance = IC_{50} resistant subline/IC_{50} parent cell line.

Table 2. Relative resistance of CDDP-resistant sublines

	CDDP	CBDCA	ADM	5-FU
A431/CDDP1	3.1	1.7	0.9	1.3
A431/CDDP2	2.7	2.6	0.6	1.0

Relative resistance = IC_{50} resistant subline/IC_{50} parent cell line.

Determination of Cross-Resistance to Other Anticancer Drugs

Although the two CDDP-resistant sublines showed cross-resistance to a CDDP analogue, CBDCA, IC_{50} of CBDCA on CDDP sublines was less than that of CDDP (CBDCA/CDDP; A431/CDDP1, 0.55, A431/CDDP2, 0.96). On the other hand, these sublines did not show cross-resistance to anticancer agents

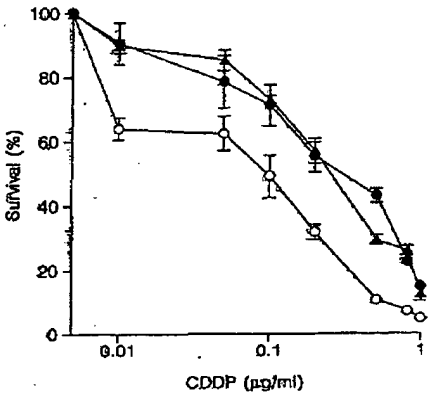

Fig. 1. Dose-response curves for CDDP in A431/P and its resistant cell lines: ○ = A431/P; ● = A431/CDDP1; ▲ = A431/CDDP2. Points and bars represent ± SD of 3 determinations.

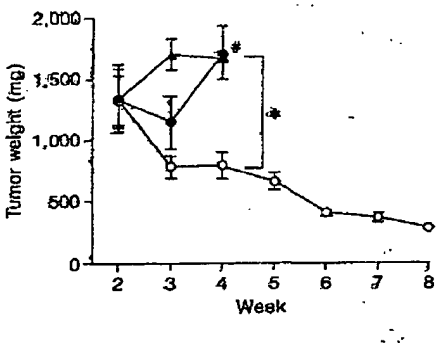

Fig. 2. Response of A431/P, A431/CDDP1 and A431/CDDP2 tumors of nude mice to serial administration of CDDP (5 mg/body weight). ○ = A431/P; ● = A431/CDDP1; ▲ = A431/CDDP2. Points and bars represent mean ± SD (n = 4 nude mice for each group). # = all mice died by 4 weeks after cell inoculation. * $p < 0.01$, significantly different from the group transplanted with parent A431 cells.

with different pharmacological action such as ADM and 5-FU (table 2). These results indicated that A431/CDDP1 and A431/CDDP2 were not multidrug resistant but specifically resistant to the CDDP drug family.

In vivo Drug Sensitivity Assay

The 1 × 10^7 cells of A431/P and CDDP-resistant sublines were inoculated subcutaneously into nude mice. The development of tumor in each group reached approximately 1,300 mg by 2 weeks after the cell inoculation. The experimental dosage of CDDP (5 mg/kg, body weight) was then administered intraperitoneally once a week until the animal died. The nude mice transplanted with the two CDDP-resistant sublines showed a significant increase of tumor growth and they also manifested cachexia. All mice transplanted with A431/CDDP1 and A431/CDDP2 died within 4 weeks after cell transplantation. On the other hand, nude mice transplanted with A431/P cells survived for 8 weeks, and tumor weight in this group was dramatically decreased by the treatment with CDDP. Thus, both CDDP-resistant sublines also presented the CDDP resistance phenomenon in vivo (fig. 2).

Intracellular Platinum Concentration

Intracellular platinum concentration time-dependently increased in all cell lines. However, the concentrations in A431/CDDP1 and A431/CDDP2 were much lower than in the A431/P cell. Incubation with CDDP for 7 h decreased the intracellular platinum concentration in A431/CDDP1 (47%) and A431/CDDP2 (25%) compared with that of A431/P. These results indicate that this CDDP resistance was dependent on the decrease of intracellular platinum accumulation (fig. 3).

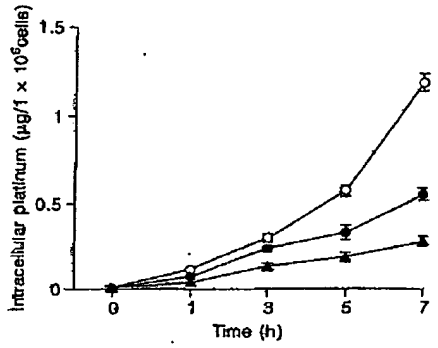

Fig. 3. Intracellular platinum concentration as a function of exposure time: O = A431/P; ● = A431/CDDP1; ▲ = A431/CDDP2. Results are expressed as platinum concentration per 1×10^6 cells. Each value represents the mean ± SD of 3 determinations. Incubation with CDDP for 7 h significantly different from the parent A431 cell ($p < 0.01$).

Discussion

CDDP has been accepted to be the most effective chemotherapeutic agent for cancer patients [1]. However, in cancer patients who had been previously treated with CDDP, the efficacy of additional CDDP therapy often decreases, since the tumor has frequently acquired resistance to this drug. This phenomenon represents a limitation of clinical cancer therapy. Epithelial carcinoma is the most common cancer in the oral region, and CDDP has been frequently used for its treatment. However, definite information about its resistance to chemotherapy is scarce due to the lack of CDDP-resistant cancer cell lines derived from mucous origin epithelial tissue. Therefore, the investigation of the function and mechanism of resistance is very important to establish strategies that may decrease CDDP resistance and increase its curative effect.

In the present study, we used A431 cells established from vulvar carcinoma to establish a CDDP-resistant cell line derived from human mucous epithelial carcinoma. Although this cell line is not an oral cancer cell line, many researchers have used it to investigate the cancer biology of oral squamous cell carcinoma [22]. Because this cell line has features similar to those seen in oral cancer. Therefore, establishment of CDDP-resistant A431 is very useful to perform further research, since the biological properties of A431 have already been clarified to a great extent.

The multiple biochemical and genetic mechanisms of CDDP resistance are not fully understood and different model systems have been proposed. Most of these models consist of highly resistant (>10-fold or more) mammalian tumor cell lines in which resistance has been induced by exposing cells continuously to increasing concentrations of CDDP for extensive periods of time [5, 23]. However, in the clinical scenario, development of resistance occurs after only a few courses of low-dosage chemotherapy. Simmonds et al. [16] and Wolf et al. [17] demonstrated in ovarian carcinomas that clinical exposures will induce low tumor CDDP resistance (3- to 6-fold). Therefore, it was our goal to mimic the clinical situation, inducing low-level CDDP resistance to low doses of CDDP and short-term exposure of the cell lines used for the in vitro and in vivo experimental procedures of this study.

The major cytotoxic action induced with CDDP has been considered to be platination of the DNA, leading to the induction of interstrand and predominantly intrastrand crosslinks [24–26]. In vitro resistance to CDDP has been shown to decrease drug accumulation in cancer cells [27]. Bungo et al. [28] reported that CDDP accumulation of a CDDP-resistant human non-small cell lung cancer cell line, PC-9/0.5, was decreased to

20% compared with that of parental PC-9. Kawai et al. [29] reported that the degree of CDDP resistance was correlated with reduction in CDDP accumulation. Teicher et al. [20] reported that both human head and neck squamous cell carcinoma cell line SCC-25 and CDDP resistance cell line SCC-25/CP concentrate platinum in the nuclei to some degree. However, there is a difference in platinum levels in the parent cell line compared to the CDDP-resistant line. In the present study, intracellular platinum accumulation in A431/CDDP1 and A431/CDDP2 decreased in contrast with parent cell line A431/P. These data suggested that reduction of intracellular platinum accumulation is one of the mechanisms profoundly involved in the resistance of both CDDP-resistant A431 cell lines.

It is well known that the acquisition of resistance to anticancer agents is dependent on multifunctional steps. Although further investigation is required, we believe that the CDDP-resistant cell lines in a nude mouse in vivo tumor model and in cells cultured in vitro observed in the present study provide of a useful model for the study of CDDP resistance.

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